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Immunology

Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection

(immune escape/polymorphonuclear cells/tumor infiltration/tumor specificity)

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ABSTRACT **During tumor progression, variants may arise that grow more vigorously. The fate of such variants depends upon the balance between aggressiveness of the variant and the strength of the host immunity. Although enhancing host immunity to cancer is a logical objective, eliminating host factors necessary for aggressive growth of the variant should also be considered. The present study illustrates this concept in the model of a spontaneously occurring, progressively growing variant of an ultraviolet light-induced tumor. The variant produces chemotactic factors that attract host leukocytes and is stimulated *in vitro* by defined growth factors that can be produced or induced by leukocytes. This study also shows that CD8$^+$ T-cell immunity reduces the rate of tumor growth; however, the variant continues to grow and kills the host. Treatment with a monoclonal anti-granulocyte antibody that counteracts the infiltration of the tumor cell inoculum by non-T-cell leukocytes did not interfere with the CD8$^+$ T-cell-mediated immune response but resulted in rejection of the tumor challenge, indicating a synergy between CD8$^+$ T-cell-mediated immunity and the inhibition of paracrine stimulation.**

Cancer cells commonly generate variants that grow more aggressively than parental cancer cells. For example, primary ultraviolet (UV) light-induced tumors in mice are often rejected when transplanted into immunocompetent naive mice (1), but such "regressor" tumors (RE tumors) give rise to variants [called "progressor" tumors (PRO tumors)], which upon transfer to normal mice do not regress but grow to kill the host. Several mechanisms of escape have been identified—for example, selective loss of the T-cell-recognized target antigen (2) or loss of the particular major histocompatibility complex class I molecule presenting the antigen (2, 3). Interestingly, however, most progressor variants of UV-induced regressor tumors selected in immunocompetent hosts must use some other mechanism to escape because these variants retain the T-cell-recognized antigen and the presenting major histocompatibility complex class I molecule (2). Recently, we have found evidence suggesting that the ability of such antigen-positive progressor variants to form tumors in normal hosts can correlate with acquisition of sensitivity to stimulation by paracrine growth factors (ref. 4; and L.P.S., unpublished work). Such factors were postulated to come from inflammatory stromal cells surrounding the cancer cells; the variants produced more chemotactic factors for inflammatory cells and were stimulated *in vitro* by growth factors produced by the inflammatory cells (4). Furthermore, elimination of cells expressing the granulocyte marker Gr-1 slowed the growth of these tumors in T-cell-deficient mice (4). We hypothesized that paracrine stimulation of growth might outstrip T-cell-mediated inhibition of tumor growth. The purpose of the present study was to test this notion and, indeed, we found that depletion of granulocytes allowed CD8$^+$ immunity to eradicate the malignant cells, thus showing an important mechanism of tumor escape and suggesting additional approaches for treatment of some cancers.

MATERIALS AND METHODS

Mice and Tumors. Five- to ten-week-old C3H nude mice, normal BALB/c, and C3H/HeN mammary tumor virus-negative (MTV$^-$) mice were purchased from the Frederick Cancer Research Facility. The 4102-RE, the 6132A-RE, and the 6134A-RE tumors were induced by UV light in C3H/HeN mice and adapted to culture as described (2, 5). These tumors are called regressors because they are rejected by syngeneic mice but grow to kill nude mice. These tumors express specific—i.e., individually distinct cytotoxic T-cell (CTL)-defined antigens. The 4102-PRO tumor variant was isolated as a rare tumor that grew in normal C3H mice. Unlike the parental 4102-RE tumor, the variant grows progressively to kill both nude and euthymic syngeneic mice. All cell lines were cultured in minimal essential medium (MEM)/10% fetal calf serum (CMEM). Tissue-cultured tumor cells were inoculated into C3H nude mice by s.c. injection. Once a solid tumor had formed, it was removed and cut into 1-mm^3 fragments that were injected s.c. into the flanks of athymic nude or C3H/HeN MTV$^-$ mice by using a 13-gauge, 7-cm-long trocar loaded with 5 or 10 1-mm^3 tumor fragments. Tumors were measured every 3–4 days. Size in cm^3 was calculated as $a \times b \times c/2$, where a, b, and c are 3 orthogonal diameters. This formula is derived from the formula for the volume of an ellipsoid: $\pi abc/6$.

Induction and Recovery of Peritoneal Exudate Lymphocytes. The 5×10^6–1×10^7 4102-RE or 4102-PRO tumor cells were injected i.p. four times at 3-day intervals. Two days after the final injection the mice were sacrificed. Peritoneal exudate lymphocytes were removed by injecting 5 ml of sterile phosphate-buffered saline, massaging the abdomen, and then aspirating the fluid. The recovered cells were sedimented at 225 $\times$ *g* for 5 min and resuspended in CMEM. Cytoxicity was measured in a 4.5-hr ^{51}Cr-release assay.

Mixed Lymphocyte-Tumor Cell Cultures. C3H/HeN MTV$^-$ mice were immunized by s.c. injections of five 1-mm^3 tumor fragments from a C3H nude mouse. Spleens were aseptically removed 22 days after tumor challenge and made into single-cell suspensions by using sterile tissue grinders. Erythrocytes were lysed with Tris·ammonium chloride (0.83%), and spleen cells were washed twice with CMEM. Tumor cells were treated with mitomycin C for 45 min at 37°C and washed three times. Cultures consisted of 8×10^6 spleen cells and 4×10^4 treated tumor cells in 3 ml of RPMI 1640 medium/10% nonheat-inactivated fetal calf serum/1% penicillin/streptomycin/0.1% gentamycin/5×10^{-5} M 2-mercap-

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Abbreviations: CTL, cytotoxic T cell(s); RE tumor, regressor tumor; PRO tumor, progressor tumor; anti-Gr-1, anti-mouse granulocyte antibody; MTV$^-$, mammary tumor virus-negative.

toethanol. Cultures were incubated for 6 days at 37°C in 16 × 125 mm round-bottom tissue culture tubes.

Assay for Allo-antigen Response. C3H/HeN MTV$^-$ mice were injected in the footpads three times at 2-day intervals with 2×10^7 BALB/c spleen cells. Other groups also received 250 μl of anti-mouse granulocyte antibody (anti-Gr-1) ascites fluid (see below) i.p. or in the footpads (four mice per group). Two days after the last injection mice were sacrificed. Spleens and lymph nodes were removed and used directly in a ^{51}Cr-release assay.

^{51}Cr-Release Assay. Cytotoxicity of CTL was determined in a 4.5-hr ^{51}Cr release assay. Effector cells from mixed lymphocyte-tumor cell cultures or peritoneal exudate lymphocytes were serially diluted in V-bottom 96-well plates in 100 μl of CMEM. Tumor cells (5×10^5) in 100 μl of CMEM were mixed with 100 μl of ^{51}Cr (sodium chromate at 1 mCi/ml; 1 Ci = 37 GBq) for 1 hr. Labeled tumor target cells were washed four times with CMEM and resuspended in CMEM at 5×10^4 cells per ml. One hundred microliters of this suspension was added to each well of effectors. Cultures were incubated for 4.5 hr at 37°C and 7.5% CO_2 in a humidified incubator. After incubation, 100 μl of supernatant from each well was collected and analyzed for radioactivity by using a γ counter (Micromedic Systems, Horsham, PA). The percentage of specific lysis was calculated with the formula: % lysis = (experimental release − spontaneous release) (maximum release − spontaneous release) × 100. Spontaneous release was ≤15% of maximum. Maximum release was determined by detergent lysis of targets.

Antibodies. Mice were depleted of Gr-1$^+$ cells by i.p. injection every 3 days with 0.2 ml of ascites fluid (≈ 200 μg of total IgG, purifiable on protein G) from nude mice bearing the IgG2b rat anti-mouse anti-Gr-1 hybridoma RB6-8C5 (from Robert Coffman, DNAX Research Institute) (6). Purified rat myeloma IgG2b (100 μg per mouse) was used as an isotype control (Zymed). Mice were depleted of CD8$^+$ cells by i.p. injection every 5 days with 0.2 ml of ascites fluid from nude mice bearing the rat anti-mouse Lyt-2 hybridoma YTS169.4.2 (American Type Culture Collection). All mice (antibody-treated and nontreated controls) received prophylactic antibiotic in drinking water (sulfamethoxazole/trimethoprin pediatric suspension at 5 ml per 200 ml of water; Geneva Pharmaceuticals, Broomfield, CO). Antibiotic treatment was started 2 days before the beginning of experiments. In the absence of prophylactic antibiotic, all mice receiving anti-Gr-1 treatment invariably die within a week; however, with prophylactic antibiotic antibody-treated mice appear vigorous and healthy. To measure granulocyte depletion, peripheral white blood cell counts and peripheral blood smears were prepared from orbital plexus venous blood. The effects of long-term anti-Gr-1 treatment were analyzed by making smears of spleen-cell suspensions of anti-Gr-1-treated 4102-PRO-tumor immune, untreated 4102-PRO-tumor immune, and naive C3H/HeN mice. A total of four anti-Gr-1 treatments were given at 3-day intervals. The last treatment was 2 days before harvesting spleens. Two to three × 10^5 spleen cells, prepared as described above for the mixed lymphocyte-tumor cell cultures, were suspended in 0.2 ml of MEM/50% fetal calf serum and spun onto glass slides in a Shanndon cytospin at 5500 rpm for 7 min. Slides were fixed and stained with Wright stain. The percentage of mature granulocytes among nucleated cells in the smears was then determined.

RESULTS

Parental Cancer Cells and Escape Variants Stimulate Comparable Tumor-Specific CTL Responses. Either 4102-RE or 4102-PRO tumor cells were injected i.p. repeatedly into normal mice. Fig. 1 shows that the parental 4102-RE and variant 4102-PRO tumor cells stimulate equivalent cytolytic anti-4102 responses to both tumor cell lines. Using peritoneal exudate lymphocytes obtained directly from the mouse avoided possible artifacts that might be introduced by culturing the lymphocytes with tumor cells *in vitro* before testing. Nevertheless, spleen cells from C3H mice immunized s.c. with either the 4102-RE or 4102-PRO tumor cells or fragments and cultured with the tumor cells generated equivalent high CTL responses measured against either tumor (data not shown).

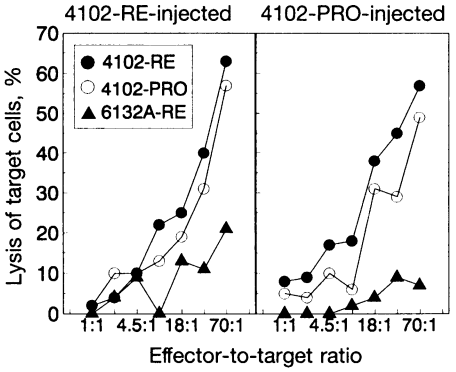

FIG. 1. The 4102-PRO tumor variant induces a tumor-specific CTL response *in vivo* as effectively as the parental cells. C3H/HeN mice received four consecutive i.p. injections of either 4102-RE tumor cells (*Left*) or 4102-PRO tumor cells (*Right*) (5×10^6–1×10^7 cells per injection per mouse) every third day. Peritoneal exudate lymphocytes were harvested 2 days after the last injection and used as effectors in a 4.5-hr cytolysis assay. 6132A-RE tumor cells were used as a control for specificity. The symbols for the targets are indicated in the inserted legend.

The Growth of the Progressor Variant *in Vivo* Is Restrained by CD8$^+$ T Cells. Immunity induced by the progressor variant might affect the rate of tumor growth, even though the tumor grows progressively. To test this possibility, we first compared the growth rates of the PRO tumor variant in nude mice and syngeneic C3H mice and found it grew significantly faster in nude mice (Fig. 2 *Left*). Secondly, we also found that treatment of normal mice with monoclonal anti-CD8 antibody resulted in an increased growth rate similar to that in the nude mice (Fig. 2 *Right*). Thus, the PRO tumor variant induces CTL immunity in normal mice, which reduces the rate of tumor growth but is not sufficient to protect against continued growth and the eventual death of the host.

Lack of a Measurable Effect of Anti-Gr-1 on CTL Responses. We previously demonstrated that the growth of the PRO tumor variant was slowed in nude mice treated with

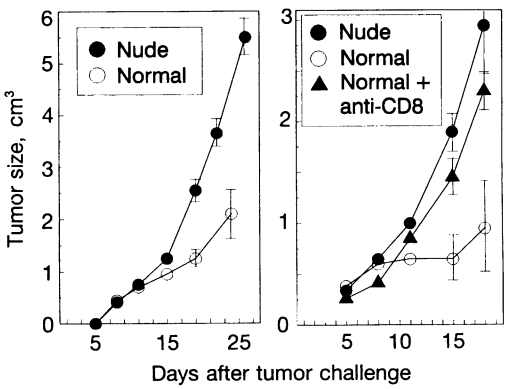

FIG. 2. T cells restrain the growth of 4102-PRO tumor *in vivo*. (*Left*) The 4102-PRO tumor grows faster in nude mice than in T-cell-competent mice. (*Right*) Treatment of euthymic mice with anti-CD8 antibody every 4 days to deplete CD8$^+$ T cells results in an increased growth rate of 4102-PRO tumor similar to that seen in nude mice. The average tumor sizes in groups of four mice ± SEM are plotted.

anti-Gr-1 monoclonal antibody RB6-8C5 (4), indicating that $\alpha\beta$ T cells were not required for this inhibitory effect. Further, while anti-Gr-1 effectively eliminates granulocytes and binds to mature myeloid cells (6), it does not bind to the tumor 4102-PRO (Fig. 3). A previous study using rats and a different monoclonal antibody to deplete neutrophils found that such treatment inhibited delayed-type hypersensitivity to sheep erythrocytes and the primary and effector phases of transplantation resistance to syngeneic rat tumors (8). We therefore determined whether the RB6-8C5 anti-Gr-1 antibody would affect the generation of CTL. In repeated experiments we failed to find any evidence that a single anti-Gr-1 antibody treatment reduced or increased CTL responses in popliteal lymph nodes of mice injected in foot pads with allogeneic cells. Also, spleen or lymph node cells of antibody-treated mice responded normally in mixed lymphocyte culture (data not shown). Finally, splenic lymphocytes, obtained from 4102-RE-tumor-immunized mice that had been treated with anti-Gr-1 antibody for 3 weeks, responded the same as lymphocytes from non-antibody-treated controls in generating 4102-specific CTL when restimulated in culture (Fig. 4). Similar results were found in several repeated experiments.

It remained a possibility that multiple injections of granulocyte-depleting antibody could lead to an overproduction of granulocytes. These newly produced granulocytes could be resistant to further depletion and might actually become cytolytic. Chronic treatment with anti-Gr-1 visibly increased spleen size, and these spleens consistently contained 20–50% more nucleated cells than spleens from untreated mice. However, the number of mature granulocytes in the spleens of chronically treated mice was decreased from 6% to 0% of the nucleated cells compared with 4102-PRO tumor-immune untreated mice, whereas the number of immature "ring-form" granulocytes was doubled from 16% to 31%. Furthermore, mice treated repeatedly with the anti-Gr-1 antibody at 3-day intervals remained free of mature granulocytes in the peripheral blood, so that there were <1% mature granulocytes before each reinjection of the antibody.

Anti-Gr-1 Antibody Treatment Leads to Regression of the Progressor Variant. The above experiments indicated that 4102-PRO tumor was restrained but not eliminated by $CD8^+$ immunity and that anti-Gr-1 treatment had no measurable effect on the generation of tumor-specific CTL. We had reported (4) that this treatment slowed the growth of 4102-PRO tumor in nude mice; thus, antibody treatment might slow tumor growth in normal mice enough to allow the T-cell-mediated immune response to become effective in eradicating the tumor challenge. C3H/HeN mice were treated every 3 days with 200 μl of anti-Gr-1 i.p. beginning on the day of tumor challenge and lasting until day 21 after tumor challenge. Table 1 shows that in three experiments the majority of normal mice treated with the anti-Gr-1 had rejected the tumors completely 1 mo after tumor challenge. Fig. 5 shows that those 4102-PRO tumors that grew in the anti-Gr-1-treated mice grew significantly slower than in untreated or isotype-control-treated mice ($P < 0.0001$). Not discernible in Fig. 5 are the four 4102-PRO tumors that were rejected by anti-Gr-1-treated mice. These tumors grew to 0.18 cm^3 ± 0.02 SEM on day 7 and were 0.06 cm^3 ± 0.01 SEM on day 10 before being completely rejected on day 21. The three tumors that eventually grew were 0.15 cm^3 ± 0.03 SEM on day 7 and 0.06 cm^3 ± 0.03 SEM on day 10 and thus were similar in size as those rejected.

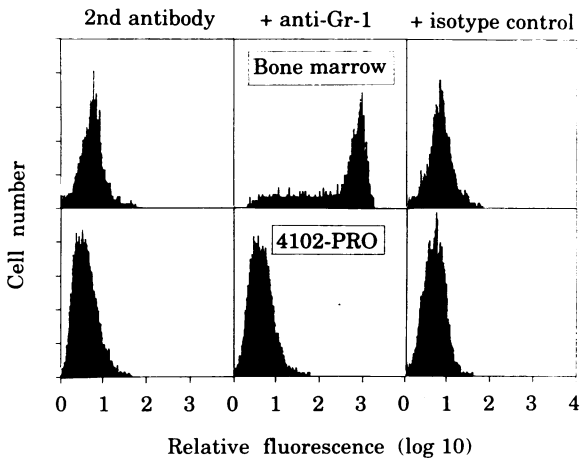

FIG. 3. Failure of the anti-Gr-1 antibody to bind to the 4102-PRO tumor cells as analyzed by flow cytometry. The same antibody binds very effectively and specifically to a subset of murine bone marrow cells at low dilution (250 ng/ml) as described (6). The isotype control antibody (7) shows no reactivity. Even at 25 μg/ml tumor cells do not react to the anti-Gr-1 antibody (data not shown). The second step antibody was a goat anti-rat IgG $F(ab')_2$ fluorescein isothiocyanate-labeled antibody (Caltag, South San Francisco, CA). Ten thousand cells were analyzed by FACSCAN II using lysis II software (Becton Dickinson).

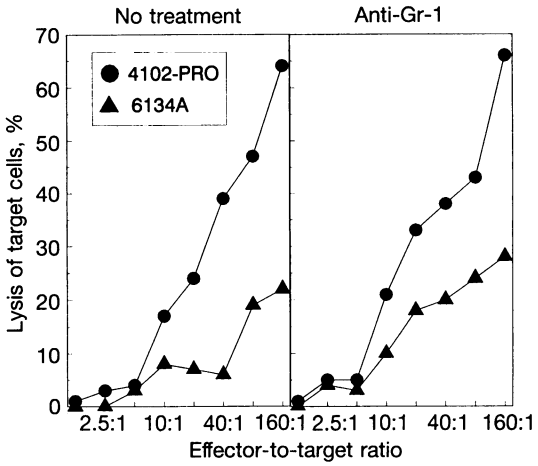

FIG. 4. Comparable cytotoxic activity of cultured spleen cells from nontreated and anti-Gr-1-treated mice against 4102-PRO tumor. C3H/HeN mice were immunized by injecting five 1-mm^3 tumor fragments of 4102-RE tumor from a C3H nude mouse s.c. Spleens were aseptically removed 22 days after tumor challenge. Spleen cells were prepared and cultured as described. Six days later a cytolysis assay was done by using as targets the 4102-PRO tumor line (●) and the unrelated syngeneic UV-induced tumor cell line 6134A (▲) as a control for specificity.

Table 1. Rejection of 4102-PRO tumor in mice treated with anti-Gr-1

Exp.	No treatment	Anti-Gr-1	Isotype control
1	0/8	5/9	ND
2*	1/4	4/5	1/4
3	0/6	4/7	0/4
Total	1/18 (6%)	13/21 (62%)	1/8 (12%)

No-treatment and isotype control groups are each significantly different from the anti-Gr-1-treated group ($P < 0.0005$ and $P < 0.035$, respectively), but not from each other ($P = 0.513$). Experiment 1 was terminated at day 28. Mice from experiments 2 and 3 were terminated on day 30 unless mice died earlier or were killed because they were moribund. The growth curves of experiment 3 are shown in Fig. 5. ND, not determined.

*Mice received five 1-mm^3 4102-PRO tumor fragments in experiment 2 and 10 1-mm^3 4102-PRO tumor fragments in experiments 1 and 3 using a trocar.

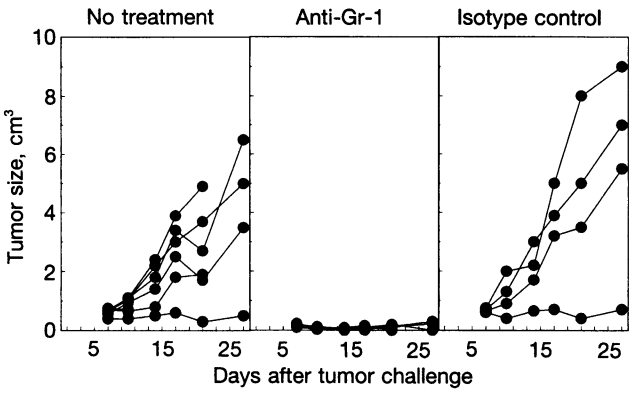

FIG. 5. Treatment of euthymic mice with anti-Gr-1 reduces the growth of 4102-PRO tumor. C3H/HeN mice were challenged with tumor fragments from a C3H nude mouse. One group received no antibody treatment (*Left*). Treated mice received either anti-Gr-1 (RB6-8C5, *Center*) or an equivalent amount of an irrelevant rat myeloma IgG2b antibody as an isotype control (*Right*) every 3 days beginning on the day of tumor challenge. Data are from experiment 3 of Table 1, which shows six nontreated mice, seven anti-Gr-1-treated mice, and four isotype control-treated mice.

DISCUSSION

We have demonstrated that the 4102-PRO tumor variant induces CTL *in vivo* as effectively as the parental 4102 cells. This tumor not only remained sensitive to lysis by specific T cells *in vitro* but could also induce a 4102-specific CTL response *in vivo*. 4102-PRO tumors grew considerably faster in the absence of $CD8^+$ T cells (in nude or anti-CD8-treated mice) than in normal syngeneic mice, indicating that T cells were responding to the tumor. Apparently, the T cells were not prevented from being induced, being recruited to the tumor site, and slowing its growth.

In a naive mouse, it normally takes at least 10 days to induce a specific CTL response to a challenge with tumor cells. The optimal time for recovering specific CTL from spleens of such mice is 2–3 weeks; this is also the time it takes for a T-cell-competent mouse to completely reject a regressor tumor, which forms a small but detectable tumor during the first 2 weeks. However, even at the peak of growth before regressing, the 4102-RE tumor is consistently smaller than the progressor variant. Furthermore, we had previously reported that 4102-PRO tumor grows significantly faster than 4102-RE tumor in nude mice, and we found evidence suggesting that this difference was due to 4102-PRO tumor having acquired sensitivity to paracrine stimulation by several growth factors (4). In particular, we found that the growth of the 4102-PRO tumor variant, but not of the parental 4102-RE tumor cells, was stimulated by recombinant transforming growth factor β, epidermal growth factor, platelet-derived growth factor, and basic fibroblast growth factor, factors either produced directly by granulocytes or produced by other infiltrative cells that are attracted and stimulated by factors released from granulocytes. At present it is not clear which factor and cell types are the ultimate paracrine stimulators. Depletion of the $Gr\text{-}1^+$ cells with a monoclonal antibody *in vivo* led to a significantly slower growth of 4102-PRO tumor in nude mice, although this treatment never led to tumor rejection by these mice (4). Such mice were not only severely depleted of granulocytes but also failed to recruit other inflammatory cells to the site of tumor injection (L.P.S., unpublished work) in agreement with previous studies of others (9, 10). Anti-natural killer antibodies and the isotype control antibody anti-CD4 did not reduce the 4102-PRO tumor growth in the nude mice (4) and the anti-Gr-1 antibody did not bind to the tumor cells. In the present study, we found that treatment of normal mice with anti-Gr-1 led not only to a significant decrease in 4102-PRO tumor growth but to complete rejection of the tumors in the majority of mice. (The fact that one of four mice in both the non-treated and isotype control-treated groups rejected the tumor in experiment 2 may be due to the smaller tumor challenge given.)

We do not know how commonly the growth of primary cancers is restrained in humans by T-cell immunity, but numerous studies have shown the presence of T cells in human tumors that can be cultured to lyse tumor cells (refs. 11 and 12; for review, see ref. 13). Furthermore, experimental animals have been shown to respond to their autochthonous cancers (14), even though prior removal of the cancer may be necessary for the immunity to become detectable. Continuous tumor growth can eclipse antitumor immunity (15, 16). Counteracting paracrine stimulation might reverse tumor-induced immune suppression and unmask existing tumor immunity. In fact, the same growth factors—e.g., transforming growth factor β might act as paracrine stimulators (17), as well as negative regulators of $CD8^+$ T-cell immunity (18).

Considerable work in a rat model has provided evidence that depletion of neutrophils with a monoclonal antibody can interfere with immune responses (8, 9, 19). Both priming and elicitation of delayed-type hypersensitivity to sheep erythrocytes were inhibited; recruitment of mononuclear leukocytes and $CD4^+$ T cells, but not $CD8^+$ T cells, into the site of delayed-type hypersensitivity was also inhibited in rats depleted of neutrophils. Nevertheless, the investigators found that depletion of neutrophils abrogated the induction of $CD8^+$ T cells that would otherwise have inhibited the growth of tumor cells (19). By contrast, the induction of $CD8^+$ responses to the tumor used here does not appear to be altered. The reasons for this discrepancy are not clear. The two antibodies might react to different types of target molecules. Furthermore, differences in the requirement for $CD4^+$ T cells for the induction of a $CD8^+$ T-cell response, could be a reason. $CD8^+$ T cells required for rejection of the particular rat tumor were induced in the presence of $CD4^+$ T cells, which alone were not effective in decreasing tumor growth. We have shown in our tumor model that $CD8^+$ but not $CD4^+$ cells are required for the rejection of the 4102-RE tumor used here and seven other UV-induced tumors tested (2).

Although we do not know how commonly the growth of human cancers depends on an environment that is influenced by $Gr\text{-}1^+$ cells, it is likely that many tumors evolve during tumor progression to a stage where they can use growth factors provided by the host in the local environment (20). We do not know the molecular basis of the heritable change that generated the 4102-PRO tumor variant, but the increased sensitivity to several growth factors and decreased serum dependence is consistent with a heritable change affecting the general growth responsiveness. A more precise understanding of the molecular events leading to paracrine stimulation will be helpful in designing more specific and powerful inhibitors. Also, we do not know which tumor types, sites, and stages of growth could be affected by interfering with paracrine stimulation, but complete rejection of the cancer cells clearly requires that tumors be antigenic and be recognized by $CD8^+$ T cells. In our model, a CTL response to the tumor is generated by the growing tumor but by itself cannot overcome the rapid growth of the tumor. In other cases, the synergistic antitumor response may first need to be induced or upregulated—e.g., by immunizing with tumor cells transfected with genes encoding cytokines (21–25), costimulatory molecules (26, 27), antisense growth factors (28), or antisense growth factor receptors (29) before tumor rejection is achieved.

Granulocytopenia occurring alone or as a component of more severe pancytopenia is a common complication of many kinds of cancer therapy. The beneficial effects of anticancer agents that suppress myelopoiesis are thought to result from

direct cytotoxicity on cancer cells, and the complications are prevented or treated with antibiotics and/or replacement therapy. Our present findings introduce the surprising concept that the complication of granulocytopenia occurring with present therapies may contribute or account for some favorable outcomes.

Producing granulocytopenia by depleting granulocytes with antibody does not affect other bone-marrow-derived cell types or B and T cells, and recovery is reversible and rapid within a few days after treatment is discontinued. Nevertheless, such antibody-induced granulocytopenia required simultaneous treatment of the host with antibiotics to reduce the risk of severe microbial infections. Therefore, it would be advantageous to identify the precise molecules and cell types directly involved in the paracrine stimulation *in vivo* so that more selective inhibitors, which have fewer or less severe potential side effects, may be developed. In any case, the results of this study support the concept that counteracting paracrine stimulation to complement the effects of existing or induced antitumor immunity may prove effective in treating some cancers.

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